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Adhesion Receptors Mediate Efficient Non-viral Gene Delivery

Inge S Zuhorn[1,2], Dharamdajal Kalicharan[3], George T Robillard[1] and Dick Hoekstra[2]

[1]Biomade Technology Foundation, Nijenborgh, Groningen, The Netherlands; [2]Department of Cell Biology/Membrane Cell Biology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands; [3]Department of Cell Biology/Electron Microscopy, Groningen, The Netherlands

For a variety of reasons, including production limitations, potential unanticipated side effects, and an immunological response upon repeated systemic administration, virus-based vectors are as yet not ideal gene delivery vehicles, justifying further research into alternatives. Unlike viral vectors, non-viral vectors pose minimal health risks, but to meet therapeutic requirements their efficacy needs major improvement. This goal may be accomplished by better defining the mechanism of non-viral gene delivery and exploiting specific cellular properties. Here we demonstrate that transfection of epithelial cells with lipoplexes is almost exclusively mediated by the β1 integrin cell surface receptor. More important, we show that in general, adhesion receptors can be exploited by lipoplexes to gain access to cells, including difficult-to-transfect primary neural stem cells and suspension cells, thereby leading to productive transfection. We propose that adhesion receptors serve as "natural" receptors for lipoplexes. As no natural cellular receptors for lipoplexes have previously been identified, our results are an important step forward in understanding the mechanisms of non-viral gene delivery. Moreover, the finding that adhesion receptors mediate efficient non-viral gene delivery paves the way for the optimization of (standard) transfection procedures as well as *ex vivo* gene therapy protocols using non-viral vectors.

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INTRODUCTION

Successful gene therapy depends on efficient and safe delivery of a transgene into the desired tissue. As viruses are by nature designed to enter cells and exploit the host cell's replication machinery, it seemed obvious to develop viral vectors for gene delivery. However, all viruses cause immune responses,[1,2] which precludes subsequent administration of the same vector. Moreover, two of the most popular viral vectors, *i.e.*, retroviruses and adeno-associated viruses, integrate into the host genome, posing the risk of insertional mutagenesis.[3,4] Non-viral vectors such as cationic lipid–DNA complexes (lipoplexes) are non-immunogenic and non-oncogenic, but their relatively low transfection efficiency compared with viral vectors is a major disadvantage.[5] The serious health concerns associated with application of viral vectors, in conjunction with options for further improvement of the rate-limiting steps in non-viral gene delivery,[6–8] have intensified research into the application of non-viral devices. In particular, current developments suggest that the need is less to synthesize novel vectors, and more to gain mechanistic insight to manipulate and hence improve transfection efficiency.

Recently, we have shown that clathrin-mediated endocytosis represents a major cellular entry pathway for lipoplexes.[9] This finding raised the question whether specific cell surface receptors are involved in lipoplex binding and/or internalization. Previously, a role for proteoglycans in transfection was suggested, but as no data were provided on binding and internalization of lipoplexes, their exact role remains enigmatic.[10] More specifically, we wondered whether the very nature of cationically charged lipoplexes of a given size, as used in our previous work, determined their effective processing by "specific" cellular receptors, in a manner analogous to the binding and internalization of a viral particle by a given cell type. Thus rather than coupling a ligand to trigger specific ligand–receptor-mediated uptake of lipoplexes, which often leads to an ambiguous outcome as it need not imply a ligand-specific internalization of such targeted complexes,[11] our aim in the current work was to identify native cellular receptors that "recognize" lipoplexes, thus exploiting cellular internalization machineries directly.

RESULTS

Loss of cell–cell contact in MDCK monolayers promotes lipoplex binding and internalization

To identify a cellular receptor for lipoplexes, we used the epithelial Madin–Darby canine kidney (MDCK) cell line. When grown to confluence on porous filters, epithelial cells form tight monolayers. The cells in these monolayers are polarized, comprising an apical (luminal) and a basolateral domain physically separated by tight junctions, each with characteristic protein and lipid compositions.[12,13] To distinguish between the involvement of apical and basolateral receptors in cellular entry of lipoplexes, MDCK monolayers were exposed to lipoplexes before and after disruption of the tight junctions with the calcium chelator ethylene glycol tetraacetic acid (EGTA). This treatment results in the loss of cell–cell contact, leading to the accessibility of (baso)lateral receptors from

Correspondence: *Inge S. Zuhorn, Department of Cell Biology/Membrane Cell Biology, University Medical Center Groningen, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands. E-mail: i.zuhorn@med.umcg.nl*

the apical side.[14] It should be noted that the addition of lipoplexes to the basolateral side of polarized MDCK cells (*i.e.*, to the basolateral chamber of a transwell filter system) does not result in binding of lipoplexes because of their gravity-induced sedimentation. Whereas lipoplex binding to the apical surface of MDCK cells is negligible, exposure of the basolateral cell surface upon loss of cell–cell contact results in massive lipoplex binding and a ninefold (from 2 to 20%) increase in transfection efficiency (**Figure 1b–e**).

To verify whether the enhancement in lipoplex binding results in a concomitant enhancement in lipoplex internalization, the lipoplex-mediated nuclear delivery of fluorescein isothiocyanate–labeled oligonucleotides (FITC-ODNs) was measured. After their uptake by cells, ODNs passively diffuse into the cell's nucleus. Therefore, the extent of nuclear accumulation of FITC-ODNs, delivered by lipoplexes, directly correlates with the extent of lipoplex internalization.[15] Incubation of EGTA-treated MDCK monolayers with lipoplexes containing FITC-ODNs results in the appearance of ODNs in essentially all nuclei, although the intensity of the fluorescent signal varies. In sharp contrast, essentially no FITC-ODN-positive nuclei are observed in control cells (**Figure 2**). Evidently, upon loss of cell–cell contact between MDCK cells, lipoplex binding and internalization are significantly enhanced, ultimately resulting in a major boost in transfection efficiency.

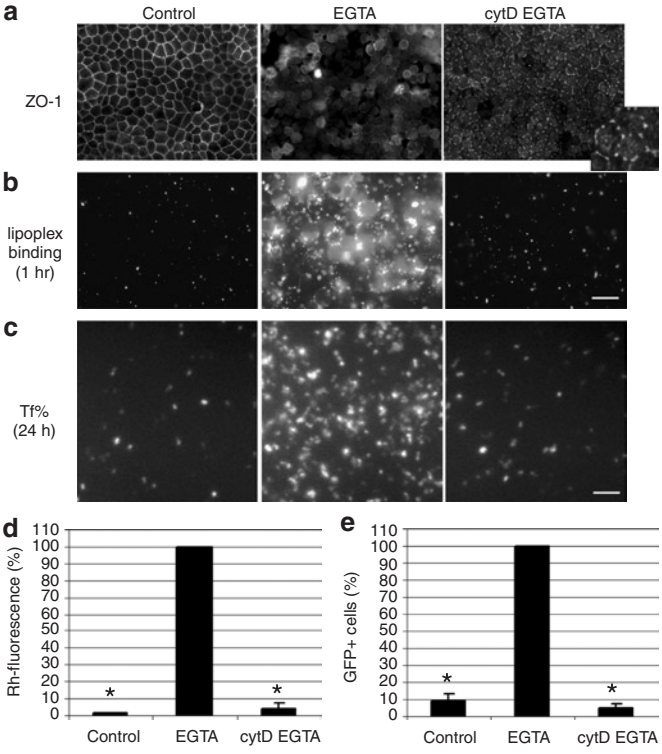

Figure 1 Lipoplex binding and transfection efficiency in Madin–Darby canine kidney (MDCK) monolayers is significantly enhanced after prior loss of cell–cell contact. (**a**) Treatment of polarized MDCK cells with ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) results in loss of cell–cell contact, which is prevented by prior disruption of actin microfilaments by cytochalasin D, as evidenced by the localization of the tight junction-associated protein ZO-1. The insert shows a close-up of the punctuate ZO-1 staining in cytochalasin D/EGTA-treated cells. The loss of cell–cell contact before the addition of lipoplexes results in a massive increase in (**b**; scale bar is 30 μm, and also applies to panels in **a**) lipoplex binding to the cells and (**c;** scale bar is 100 μm) transfection efficiency. Lipoplex binding (**d**) (with SAINT-2/DOPE) and transfection efficiency (**e**) (with Lipofectamine 2000) were quantified as indicated in Materials and Methods. Results with EGTA-treated cells were set as 100%. An asterisk indicates statistical significance compared with the EGTA condition (*t*-test; $P < 0.05$).

Typically, after tight junction disruption, lipoplexes accumulate at the cell surfaces of adjacent MDCK cells, *i.e.*, the lateral plasma membrane domain (**Figure 1b**). These sites were visualized by electron microscopy (**Figure 3b** and **c**), and lipoplexes appeared to accumulate at the cell surface near/into coated pits (**Figure 3c**, arrows), consistent with lipoplex internalization via clathrin-mediated endocytosis.[9] In **Figure 3d**, the fingerprint structure that is typical for lipoplexes visualized by electron microscopy is clear. To obtain some mechanistic insight into the effect of tight junction disruption on the process that promotes lipoplex transfection, we took into account the fact that tight junction disruption involves actin cytoskeletal rearrangements.[16] We examined the effect of the actin filament–disrupting drug cytochalasin D (cytD) in the context of the EGTA-induced enhancement in lipoplex binding and transfection. As can be appreciated from **Figure 1a**, polarized MDCK cells show a continuous circumferential pattern of the tight junction protein ZO-1, which indicates the presence of functional tight junctions. Upon EGTA treatment, ZO-1 redistributes in part into the cytoplasm, coinciding with a loss of cell–cell contact (**Figure 1a**, EGTA). Prior disruption of the actin cytoskeleton by cytD maintains the lateral distribution of ZO-1 upon EGTA treatment, although in a punctuate rather than continuous pattern (**Figure 1a**, cytD EGTA). Under these conditions, the typical rounding-up of cells is prevented, and (consequently) the enhancement in lipoplex binding and transfection efficiency (**Figure 1b–e**). These data indicate that the physical separation of cells, which is

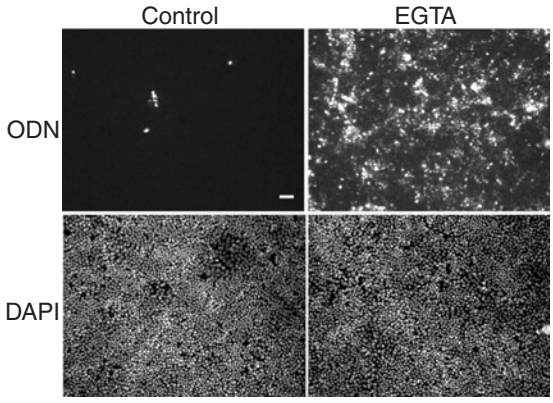

Figure 2 Prior loss of cell–cell contact between Madin–Darby canine kidney (MDCK) cells increases the internalization of lipoplexes. After the internalization of lipoplexes containing oligonucleotides (ODNs) and their subsequent endosomal escape, ODNs passively diffuse into the cell's nucleus. Therefore, the nuclear accumulation of fluorescein isothiocyanate (FITC)–labeled ODNs directly correlates with the extent of lipoplex internalization. Incubation of ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA)–treated MDCK monolayers with Lipofectamine 2000 lipoplexes containing FITC-ODNs results in the appearance of FITC-ODNs in essentially all nuclei, although the intensity of the fluorescent signal varies. In sharp contrast, essentially no FITC-ODNs are observed in monolayers that were not pre-treated with EGTA. Nuclei counterstained with DAPI are shown in the bottom row. Scale bar = 50 μm.

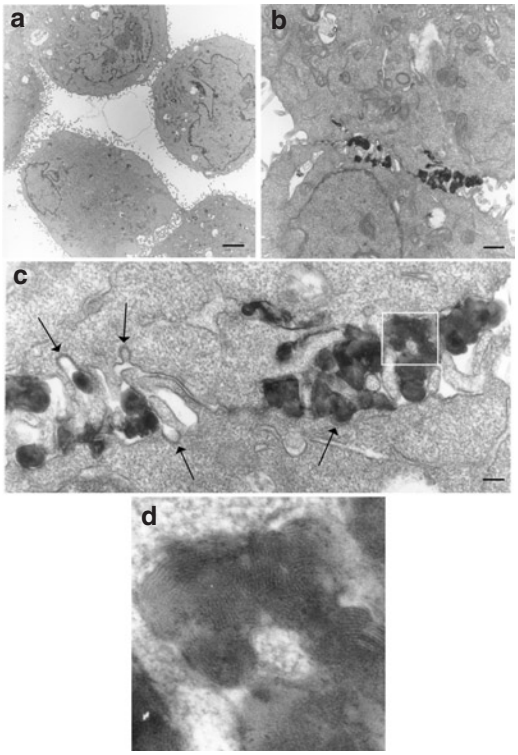

Figure 3 Transmission electron microscopy of ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA)–treated Madin–Darby canine kidney (MDCK) monolayers incubated with lipoplexes. (**a**) EGTA-treated MDCK monolayers lose cell–cell contact and display a rounded morphology (scale bar = 2 μm). (**b**) Added Lipofectamine 2000 lipoplexes (which are electron-dense) accumulate between the neighboring cells, at the sites of prior cell–cell contact (scale bar = 560 nm). (**c**) An enlargement of the accumulation site of lipoplexes in **b**, revealing the presence of lipoplexes near/in coated pits (arrows) (scale bar = 280 nm). (**d**) An enlargement of the lipoplexes in **c** (boxed area), revealing the typical fingerprint structure of lipoplexes.

dependent on an intact actin cytoskeleton, is a prerequisite for the strong promotion of lipoplex binding.

AIIB2 blocking antibody inhibits lipoplex binding and transfection efficiency in EGTA-treated MDCK monolayers

The binding pattern of lipoplexes to EGTA-treated MDCK monolayers indicates binding to receptors that are localized at the lateral plasma membrane. Two adhesion molecules that are enriched at the lateral membrane of MDCK cells, and to which function-blocking antibodies against canine origin are available, are E-cadherin and β1 integrin.[17,18] To investigate their potential involvement in lipoplex internalization, we investigated whether function-blocking antibodies could abolish the enhancement in transfection efficiency of EGTA-treated MDCK cells. Neither rr1 and rbαE-cadherin (both blocking antibodies against E-cadherin) nor RGD peptide (which primarily binds αv integrins) affected the transfection efficiency of MDCK cells after EGTA treatment (**Figure 4a**). Likewise, P2A4 antibody against human (not canine) ICAM-1 (an aspecific isotype control) did not affect the transfection efficiency. In contrast, AIIB2 blocking antibody against β1 integrin almost completely abolished the transfection efficiency in EGTA-treated monolayers (**Figure 4a**), and the extent of lipoplex binding was reduced by approximately 50% under the same conditions (**Figure 4b**). These data suggest that multiple binding entities may exist on the cell surface but that those represented by the pool of β1 integrins play a prominent role in the internalization of lipoplexes leading to productive transfection. Interestingly, calcium is known to promote the bent (low-affinity) conformation of integrins.[19] In contrast to the bent conformation of integrins, in which their ligand-binding site orients toward the plasma membrane, integrins in the high-affinity conformation have been suggested to extend far above the surface of a cell, which will likely facilitate ligand binding. We therefore assume that (positively charged) lipoplexes bind extensively to the (negatively charged) regions that become exposed upon integrin activation. Subsequently, lipoplexes piggyback along with the integrins that become internalized upon activation and/or ligand (lipoplex) binding.

In EGTA-treated MDCK monolayers lateral β1 integrin redistributes along the entire plasma membrane

To verify whether tight junction disruption by EGTA treatment results in the recruitment of laterally localized β1 integrin receptors, the surface topography of the receptor in control and EGTA-treated MDCK monolayers was investigated by immunofluorescence. In control cells, permeabilized after fixation, β1 integrin is mainly localized laterally (**Figure 5a**, cf. ref. 20). After EGTA treatment, the exclusive lateral distribution of β1 integrin is lost and the receptor redistributes at least partly to the entire plasma membrane (**Figure 5a**, EGTA). In MDCK cells pre-treated with cytD, β1 integrin largely remains laterally distributed, but can also be found at the limiting membrane of large vesicular cytoplasmic structures (**Figure 5a**, cytD EGTA). Importantly, in non-permeabilized MDCK cells, β1 integrin could be revealed only after EGTA treatment (data not shown). Taken together, the data indicate that apical membranes are devoid of the receptor; given that β1 integrin receptors at the lateral domain (*i.e.*, between cells) are not accessible to (small) antibodies in non-permeabilized polarized cells, it is similarly highly unlikely that (large) lipoplexes (200–400 nm) are able to penetrate between neighboring cells to reach their receptors. Accordingly, in conjunction with the data showing an almost complete abolishment of transfection of the cells upon treatment with the β1 integrin blocking antibody (**Figure 4a**), these observations readily explain the relatively poor transfectability of polarized epithelial cells in monolayer culture.

Transfection of MDCK monolayers after loss of cell–cell contact is cation dependent

To obtain further support for the role of integrins in lipoplex-mediated transfection, we examined the extent to which divalent cations affected transfection efficiency. It has been well established that integrin activity is modulated by divalent cations, including Ca^{2+}, Mg^{2+}, and Mn^{2+},[21] which relies on divalent cation–induced changes in its conformational state.[19,22] For example, Ca^{2+} and Mn^{2+} have been shown to act as inhibitor and activator, respectively, of β1 integrin activity.[22,23] We therefore determined the transfection efficiency of EGTA-treated MDCK cells, after a wash with Ca^{2+}, Mg^{2+}, or Mn^{2+}. As shown in **Figure 4c**, whereas Mn^{2+} sustained the enhancement in transfection efficiency of EGTA-treated cells, both Ca^{2+} and Mg^{2+} strongly inhibited transfection (**Figure 4c**).

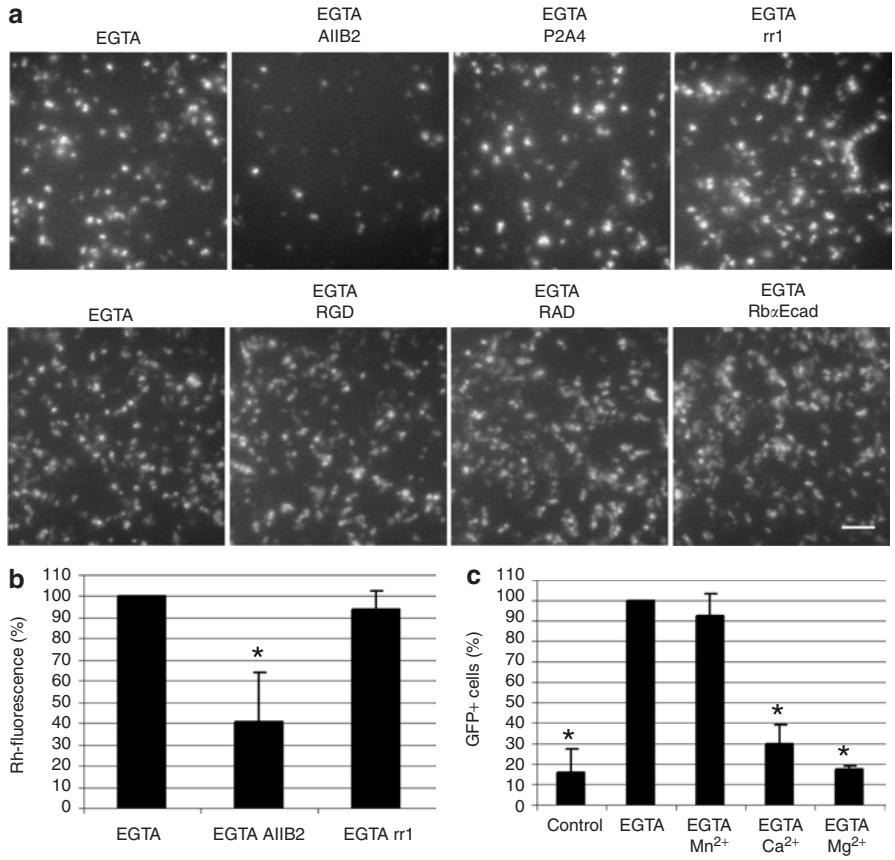

Figure 4 AIIB2 β1 integrin blocking antibody inhibits lipoplex binding and transfection efficiency in ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA)–treated Madin–Darby canine kidney (MDCK) monolayers. (**a**) Incubation of EGTA-treated MDCK monolayers with the β1 integrin blocking antibody AIIB2 inhibits transfection, whereas incubation with RGD/RAD peptides, the E-cadherin blocking antibodies rr1 and RbαEcad, and P2A4 ICAM-1 blocking antibody (which does not recognize canine ICAM-1 and thus serves as a negative control for steric hindrance of antibodies as such) are without effect. (**b**) Binding of (SAINT-2/DOPE) lipoplexes is significantly reduced (*t*-test; $P < 0.05$) in cells incubated with AIIB2 antibodies when compared with cells incubated with rr1 antibodies (EGTA condition set as 100%). (**c**) Whereas Mn^{2+} sustains transfection with Lipofectamine 2000 lipoplexes in EGTA-treated cells, Ca^{2+} and Mg^{2+} inhibit transfection. Transfection efficiency after EGTA treatment set as 100%. Asterisks indicate statistical significance when compared with the EGTA condition (*t*-test; $P < 0.05$). Scale bar in **a** = 100 μm.

Evidently, these effects are entirely consistent with the stimulatory and inhibitory effects on β1 integrin receptor activity of Mn^{2+}, on the one hand, and Ca^{2+}, on the other, supporting the involvement of integrins in lipoplex-mediated transfection of MDCK cells after EGTA treatment.

Down-regulation of cell surface β1 integrin expression inhibits transfection of MDCK cells

As an alternative approach to determining the role of β1 integrin in lipoplex-mediated transfection we used RNA interference. MDCK cells were treated with small interfering RNA (siRNA) directed against canine β1 integrin (sicanb1), and, as controls, murine β1 integrin (simurb1) and influenza hemagglutinin. Cells treated with sicanb1 formed seemingly perfect confluent monolayers (**Figure 5b**), but, as anticipated, cell–cell interactions were weakened, as reflected by a diminished value of the transepithelial resistance compared with control cells (**Figure 5b**). Indeed, within the monolayers, cell areas with a significant reduction in β1 integrin expression were clearly visible by immunofluorescence microscopy (**Figure 5d**). Consistent with this observation, quantitation by means of immunoprecipitation of biotinylated β1 integrin revealed that the total cell surface expression of β1 integrin in siRNA-treated cells was inhibited by 70% compared with control cells (**Figure 5e**). Importantly, the transfection efficiency of β1 integrin–depleted monolayers after sicanb1 treatment was reduced by 80% compared with cells treated with the control siRNA simurb1 (**Figure 5c**). Cells treated with siRNA against hemagglutinin showed some inhibition in transfection, but the inhibition seen in this case was statistically insignificant. As can be seen from the nuclear staining in **Figure 5d**, all siRNA-treated monolayers show some delay in growth compared with control cells. However, no specific effects of sicanb1 treatment on cell growth or viability were observed under these conditions.

Exposure of adhesion receptors promotes transfectability of a variety of cell types

As neural stem cells can be grown *ex vivo* in clusters, in which the cells display extensive cell–cell adhesion,[24] a similar protocol for improving surface exposure of receptors was applied as for MDCK cells. Interestingly, as shown in **Figure 6a**, after EGTA treatment, the transfection efficiency of neural stem cells was substantially increased. The number of transfected cells and the

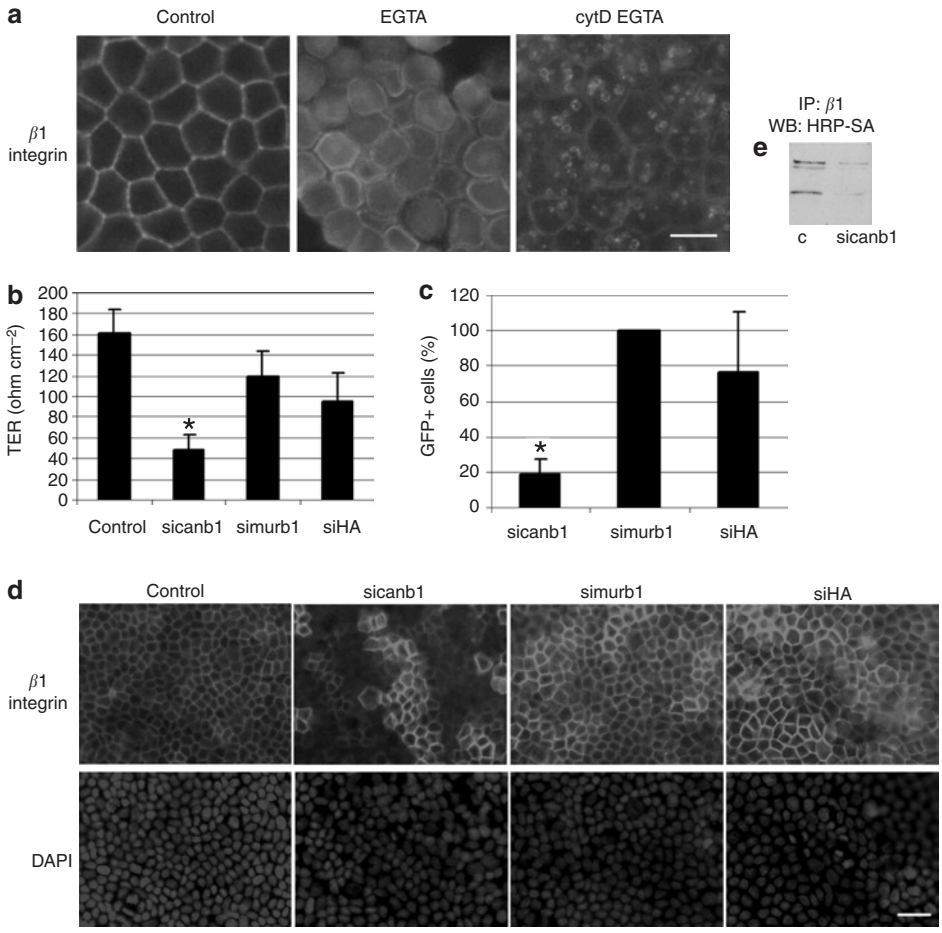

Figure 5 Interference RNA (RNAi)–mediated down-regulation of β1 integrin expression inhibits transfection efficiency in Madin–Darby canine kidney (MDCK) monolayers. (**a**) In polarized MDCK cells, β1 integrin is predominantly localized at the lateral surface (control). Upon treatment with ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), β1 integrin redistributes along the entire cell surface (EGTA). This is prevented by prior treatment with cytochalasin D (cytD), which causes the partial internalization of β1 integrin into large vacuolar structures (cytD EGTA). Size bar indicates 10 μm. (**b**) Down-regulation of β1 integrin expression by RNAi (sicanb1) reduces the transepithelial resistance (TER) of the MDCK monolayer when compared with cells treated with the control siRNAs simurb1 and influenza hemagglutinin (siHA). (**c**) Down-regulation of β1 integrin expression by RNAi (sicanb1) reduces transfection efficiency with Lipofectamine 2000 lipoplexes when compared with cells treated with the control siRNAs simurb1 and siHA (*t*-test, $P < 0.05$). Treatment of MDCK cells with sicanb1, but not simurb1 or siHA, reduces surface expression of β1 integrin, as evidenced by (**d**) immunostaining (scale bar = 30 μm) and (**e**) immunoprecipitation of biotinylated β1 integrin.

extent of reporter gene expression per cell were enhanced, which is consistent with an increase in receptor accessibility and lipoplex internalization upon loss of cell–cell contacts.

Transfection of EL4 T lymphoma and S180 sarcoma cells was not significantly altered upon EGTA treatment (data not shown). This result was not unexpected, as these cells typically grow in suspension as single cells, implying that these cells are essentially not engaged in cell–extracellular matrix (ECM) or cell–cell adhesion interactions (**Figure 6b** and **c**). To stimulate artificially cell surface recruitment of cell adhesion molecules, EL4 cells were grown in the presence of the ECM protein laminin and briefly stimulated with phorbol 12-myristate 13-acetate (PMA) before transfection. In the presence of laminin, EL4 cells readily formed large homotypic cell aggregates that remained in suspension (**Figure 6b**). Additional treatment with PMA stimulated EL4 cell activation, as evidenced by the secretion of the pro-inflammatory cytokine interferon-γ. Indeed, incubation of EL4 cells with laminin and PMA resulted in high levels of interferon-γ secretion (68.5 pg/ml) as measured 24 hours after stimulation, whereas incubation with either laminin or PMA alone resulted in low interferon-γ secretion (<10 pg/ml). Similar results were obtained when collagen was used instead of laminin (data not shown). Importantly, upon addition of PMA, EL4 aggregates became less tight, allowing for the penetration of lipoplexes between cells. We compared the transfection efficiencies of PMA-stimulated EL4 cells in the presence and absence of laminin. As shown in **Figure 6b**, activated (LN/PMA) EL4 cells displayed a fourfold increase in transfection efficiency relative to non-activated (PMA) cells. Moreover, the increase in transfection efficiency could be inhibited by RGD peptides (**Figure 6b**, graph), indicating the involvement of integrins.[25,26]

Finally, because incubation in the presence of *cis*-polyunsaturated fatty acids has been said to activate integrins and enhance cell adhesion of tumor cells,[27] we incubated S180 cells with linoleic acid before transfection. In the presence of linoleic acid, S180 cells formed large cell clusters, indicating the induction of cell–cell adhesion by linoleic acid (**Figure 6c**). These clusters

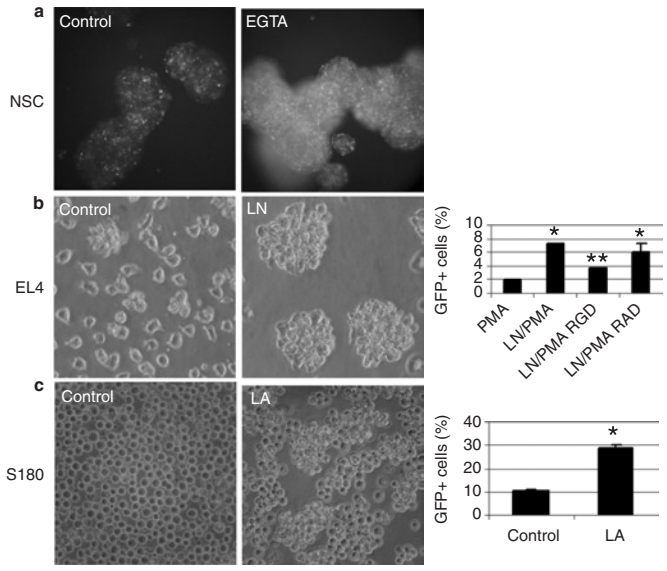

Figure 6 Activation of cell adhesion receptors enhances transfection efficiency of neural stem cells, EL4 T lymphocytes, and S180 sarcoma cells. (**a**) Incubation of ethylene glycol-bis(2-aminoethyl-ether)-*N,N,N′,N′*-tetraacetic acid (EGTA)–treated neurospheres with Lipofectamine 2000 lipoplexes results in a significant increase in green fluorescent protein (GFP)–positive cells when compared with untreated neurospheres. (**b**) Incubation of EL4 T lymphocytes in the presence of laminin results in the formation of large cell clusters that are significantly better transfected with Lipofectamine 2000 lipoplexes after phorbol 12-myristate 13-acetate (PMA) stimulation when compared with cells incubated in the absence of laminin (graph; an asterisk indicates statistical significance compared with PMA-treated cells). The increased transfection efficiency in EL4 cells treated with laminin/PMA is prevented by pre-incubation with RGD peptides (graph; a double asterisk indicates statistical significance compared with LN/PMA-treated cells). (**c**) S180 sarcoma cells form clusters in the presence of linoleic acid that show a threefold increase in transfection efficiency with Lipofectamine 2000 lipoplexes when compared with cells transfected in the absence of linoleic acid (graph).

were significantly (threefold) better transfected than single cells (**Figure 6c**, graph).

DISCUSSION

The data presented demonstrate that lipoplex-mediated transfection of MDCK monolayers can be mediated by a specific class of adhesion molecules, *i.e.*, β1 integrin receptors, made accessible upon EGTA treatment. The "specificity" of the receptor is highly intriguing, as transfection is virtually abolished when it is blocked by a specific antibody, AIIB2, and lipoplex binding is reduced by approximately 50% under the same conditions. These data suggest that multiple binding entities may exist on the cell surface but that those represented by the pool of β1 integrins play a prominent role in the internalization of lipoplexes leading to productive transfection. In future work it will be of interest to improve the transfection efficiency by means of targeting of lipoplexes to β1 integrin receptors. Future studies are also required to investigate the mechanism involved in the endocytotic internalization of β1 integrin receptors. In sub-confluent MDCK cells, transfection was strongly inhibited by prior treatment of the cells with methyl-β-cyclodextrin and chlorpromazine, indicating the involvement of clathrin-mediated endocytosis.[9] However, in confluent cells, these inhibitors could not be used as the loss of cell–cell contact induced by EGTA results from the endocytosis of cell junctional receptors such as E-cadherin.[28] Prior treatment with methyl-β-cyclodextrin or chlorpromazine would interfere with the endocytosis of E-cadherin, preventing the loss of cell–cell contact. Likewise, incubation of MDCK cells with EGTA at 10 °C (a non-permissive temperature for endocytosis) did not result in loss of cell–cell contact (I.S.Z., unpublished results), indicating that energy-dependent processes, such as endocytosis, are responsible for the rounding-up of cells upon EGTA treatment.

To investigate the generality of the observation that adhesion receptors serve as "natural" cell surface receptors for lipoplexes, we set out to challenge adhesion receptors in difficult-to-transfect primary cells and suspension cells. As (primary) neurospheres display extensive cell–cell contact, these cells could be similarly treated with EGTA to make adhesion receptors accessible to lipoplexes. Indeed, in EGTA-treated neurospheres transfection efficiency was significantly enhanced (**Figure 6a**). EL4 and S180 suspension cells, which do not display cell–cell adhesion, could be artificially stimulated to recruit and/or activate cell adhesion molecules to their cell surface when grown in the presence of specific ECM proteins and lipids, respectively. After cell surface recruitment of adhesion receptors in these cells, transfection efficiency was significantly enhanced (graphs in **Figure 6b** and **c**). Although beyond the scope of this article, it would be of interest to show which adhesion receptors mediate lipoplex internalization in the different cell types. In EL4 cells the αLβ2 integrin receptor would be a likely candidate.

Notably, in MDCK cells transfection was not inhibited by RGD peptide, whereas in EL4 cells RGD peptide did inhibit transfection. This indicates that the different treatments in the different cell types lead to expression/exposure of different (sets of) adhesion receptors. It seems contradictory for laminin to induce RGD-dependent adhesion in EL4 cells, as laminin generally binds to different subsets of integrins (α3, α6) than the ones inhibited by RGD (α5, αv). However, it should be noted that in our experimental set-up, the presence of laminin induces cell–cell contact and not cell–ECM (LN) contact; *i.e.*, laminin is a non-adhesive substrate. Also, it has been reported in the literature that laminin contains an RGD motif.[29,30]

Although the expression pattern of adhesion receptors will likely vary among various cell types and at the moment we do not exclude the potential involvement of other classes of adhesion receptors besides integrins (*i.e.*, immunoglobulins, selectins, and cadherins), our study clearly demonstrates that the accessibility/recruitment of adhesion receptors on the cell surface can be employed to achieve efficient transfection of cells that are known to be highly resistant to transfection, such as polarized epithelial cells.

Furthermore, the data suggest an apparent and interesting parallel between cellular "receptors" for entry of viruses and for entry of particles such as lipoplexes, possibly driven by charge and size. It is apparent that the current approach will be particularly relevant to *ex vivo* gene therapy as it offers a relatively simple means greatly to improve transfection efficiency of cells, including stem cells, whose therapeutic impact may have been limited thus far owing to poor transfection susceptibility.

MATERIALS AND METHODS

Cell culture. MDCK cells were grown in Dulbecco's modified Eagle's medium (Gibco, Breda, The Netherlands) containing 10% fetal bovine serum, 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Invitrogen), and 100 mg/ml streptomycin (Invitrogen) at 37 °C and 5% CO_2. Cells were plated at 2×10^5 cells/cm^2 in Transwell plates from Costar (Corning Life Sciences, Acton, MA). The next day, the cell culture medium was refreshed. At day 3 after plating, cell resistance was measured with a Millicell-ERS device (Millipore, Billerica, MA) and experiments were performed.

Neural stem cells, isolated from murine brain, were grown as neurospheres in neurobasal medium (Gibco) supplemented with 2% B27 (Gibco), 1% glutamax-1 (Invitrogen), 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Invitrogen), 100 mg/ml streptomycin (Invitrogen), 20 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen), and 20 ng/ml epidermal growth factor (Invitrogen).

EL4 cells (murine T-lymphocytes) were grown in Iscoves modified Dulbecco's medium with glutamax-1 (Gibco) and 10% fetal bovine serum.

S180 sarcoma cells were grown in Dulbecco's modified Eagle's Medium containing 10% fetal bovine serum, 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Invitrogen), 100 mg/ml streptomycin (Invitrogen), and 1 mM sodium pyruvate (Gibco).

Lipoplex binding and internalization. Three-day-old MDCK cultures were treated with the calcium chelator EGTA (2 mM, Sigma, St. Louis, MO) until cell–cell contact was lost (typically 15–45 minutes at 37 °C). Then cultures were washed with ice-cold serum-free medium (Dulbecco's modified Eagle's medium) and cooled. Subsequently, rr1 (anti-E cadherin) and AIIB2 (anti-β1 integrin) blocking antibody (Developmental Studies Hybridoma Bank, University of Iowa, IA; 10 μg/ml; 0.1% bovine serum albumin) were added for 30 minutes on ice. Lipoplexes with a positive: negative charge ratio of 2.5:1 (average size 200 nm), composed of *N*-rhodamine-PE-labeled (0.5 mol%) SAINT-2/DOPE (synthesized according to Meekel *et al.*[31]) and pEGFP-N1 (Clontech, Mountain View, CA), were added and left with the cells for 1 hour on ice (binding). Cells were extensively washed (three times) with ice-cold Hank's buffered salt solution (Gibco), filters were excised, and lipoplex binding was measured with fluorescence microscopy (Olympus, Tokyo, Japan) and quantified using Scionimage (Scion Corporation, Frederick, MA) software. SDs were calculated from at least three separate experiments performed in duplicate. Statistical significance was determined using the Student's *t*-test. Experiments in which we used Lipofectamine 2000 with FITC-ODNs gave similar results. In addition, the effect of EGTA on lipoplex internalization and transfection efficiency of MDCK cells is comparable to SAINT-2/DOPE and Lipofectamine 2000. For internalization, lipoplexes composed of Lipofectamine 2000 and FITC-ODNs (Biognostik, Göttingen, Germany) were added to cells for 1 hour at 37 °C (internalization). Cells were extensively washed with ice-cold Hank's buffered salt solution (Gibco), filters were excised, and nuclear accumulation of FITC-ODNs was visualized with fluorescence microscopy (Olympus).

Transfection of MDCK cells and neurospheres. Three-day-old MDCK cultures were incubated with lipoplexes (made in serum-free medium) composed of Lipofectamine 2000 (Invitrogen) and pEGFP-N1 (Clontech) at 37 °C, according to the manufacturer's protocol and typically adding 0.5 μg of plasmid DNA per well. The transfection efficiency was determined the next day. Cells were fixed with 4% paraformaldehyde (Sigma) and nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma). For each condition, 5,000 cells were counted. Transfection efficiency is expressed as the number of green fluorescent protein–positive cells per 5,000 cells. Before lipoplex incubation, cells were treated with 2 mM EGTA until the loss of cell–cell contact. cytD (Sigma) was used to disrupt actin microfilaments at 1 μg/ml. After EGTA treatment, cells were incubated with blocking antibodies (10 μg/ml; 0.1% bovine serum albumin) and GRGDSP (RGD) and GRADSP (RAD) peptides (200 μg/ml; Calbiochem, Nottingham, UK) for 1 hour (on ice) or washed with freshly prepared 2 mM Ca^{2+}, Mg^{2+}, or Mn^{2+}. SDs were calculated from at least three separate experiments performed in duplicate. Statistical significance was determined using the Student's *t*-test. Similar to MDCK cells, neurospheres were incubated with 2 mM EGTA before the addition of lipoplexes. Transfection efficiency was measured the next day.

Transfection of EL4 cells. EL4 cells were plated on ECM-coated 12-well plates 1 day before transfection. Plates were coated with laminin (10 μg/ml; Sigma), collagen (5 μg/ml; Boehringer-Mannheim, Germany), fibronectin (10 μg/ml; Sigma), and poly-ornithine (0.01%; Sigma). After stimulation with PMA (50 ng/ml; 30 minutes; Sigma), cells were incubated with lipoplexes for 4 hours (0.5 μg plasmid DNA/well). Optionally, EL4 cells were incubated with RGD and RAD peptide (200 μg/ml) before the addition of lipoplexes. The next day transfection efficiency was determined by FACS analysis (Elite, Coulter, Hialeah, FL; $\lambda_{ex.}$ 488 nm, $\lambda_{em.}$ 530 nm, 5,000 events). SDs were calculated from at least three separate experiments performed in duplicate. Statistical significance was determined using the Student's *t*-test

Transfection of S180 sarcoma cells. S180 cells were plated at 2×10^5 cells/ml and incubated with 200 nmol/ml linoleic acid methyl ester (Merck, Darmstadt, Germany) for 24 hours before transfection. Lipoplexes composed of Lipofectamine 2000 and containing 1 μg plasmid DNA were added. Transfection efficiency was determined the next day by fluorescence microscopy. SDs were calculated from at least three separate experiments performed in duplicate. Statistical significance was determined using the Student's *t*-test

Antibodies. Blocking antibodies against β1 integrin (AIIB2), E-cadherin (rr1), and ICAM-1 (P2A4) were obtained from the Developmental Studies Hybridoma Bank. RbαEcadherin was a gift from Dr. M. Wheelock. The antibody directed against ZO-1 was from Zymed (San Francisco, CA). Alexa fluor 488 and 568 secondary antibodies were purchased from Molecular Probes (Eugene, OR).

siRNA treatment of MDCK cells. siRNA duplexes against canine (XM_535143) and murine β1 integrin (NM_010578) were synthesized by Eurogentec (Seraing, Belgium). Sequences of siRNA duplexes were 5′ GCUC CAGCCAGAAGAUAUUdTdT 3′ and 5′ GCAUUGGCUUUGGCUCAU UdTdT 3′ for canine and murine β1 integrin, respectively. siRNA against hemagglutinin (5′ UAUGCGACAGUCCUCCUCACCAdTdT 3′) was a gift from Dr. A. Huckriede (University of Groningen, The Netherlands). MDCK cells were treated with siRNA (80 nM) on day 1 and day 2 after plating, using Lipofectamine 2000 according to the manufacturer's instructions. (Note that trypsinization of sicanb1-treated MDCK cells rendered the cells with a diminished capacity to restore attachment to a cell culture dish/filter. Therefore, cells were treated twice with siRNA without replating.) On day 3 the transepithelial resistance was measured (Millicell-ERS, Millipore) and experiments were performed. Down-regulation of β1 integrin level was determined by immunostaining and immunoprecipitation, using the AIIB2 antibody.

Immunoprecipitation of β1 integrin. siRNA-treated MDCK monolayers were treated with EGTA, cooled on ice, and biotinylated (EZ-link Sulfo-NHS-LC-Biotin; Pierce Biotechnology, Rockford, IL) for 30 minutes on ice. Subsequently, cells were lysed (cold) in 1% Triton X-100, supplemented with protease inhibitors (Complete Mini protease inhibitor cocktail; Roche, Indianapolis, IN). β1 integrin was immunoprecipitated, using AIIB2 antibody coupled to protein G-sepharose beads, following standard procedures. Beads containing antibody–antigen complexes were re-suspended in sample buffer (reducing) and boiled for 5 minutes. Beads were spun down, and supernatants were applied onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%), followed by transfer

onto nitrocellulose membranes. Membranes were blocked with 5% milk in Tris-buffered saline Tween-20 and incubated with streptavidin-horseradish peroxidase. Blots were processed for enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, UK) detection. Bands were quantified using Scionimage software.

Transmission electron microscopy of EGTA-treated MDCK cells. After EGTA treatment, MDCK cells were cooled on ice and incubated with lipoplexes for 1 hour. Subsequently, cells were warmed to 37 °C and incubated for 10 minutes to initiate internalization of lipoplexes. Cells were fixed for 1 hour on ice in 1.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, containing 1% sucrose. After postfixation in 1% OsO_4/1.5% $K_4Fe(CN)_6$, cells were dehydrated in graded alcohol series and embedded in Epon 812. After polymerization for 4 days at 45 °C, ultra-thin sections were cut and stained with 1% tannic acid and 1% uranylacetate. (All chemicals used for the processing of cells for investigation by transmission electron microscopy were from Sigma.) The sections were examined using a Philips CM 100 electron microscope (Eindhoven, The Netherlands) operating at 60 kV and micrographs were taken.

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